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Human fetal liver metabolism of oxycodone is mediated by CYP3A7

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Abstract

Oxycodone is an opioid analgesic that is commonly prescribed to pregnant women to treat moderate to severe pain. It has been shown to cross the placenta and distribute to the fetus. Oxycodone is mainly metabolized by CYP3A4 in adult liver. Since CYP3A7 is abundantly expressed in the fetal liver and has overlapping substrate specificity with CYP3A4, we hypothesized that the fetal liver may significantly limit fetal exposure to oxycodone. This study showed that oxycodone is metabolized by CYP3A7 to noroxycodone in fetal liver microsomes (FLMs). The measured CYP3A7 expression was 191–409 pmol/mg protein in 14 FLMs and an intersystem extrapolation factor (ISEF) for CYP3A7 was 0.016–0.066 in the panel of fetal livers using 6β-OH-testosterone formation as the probe reaction. Noroxycodone formation in fetal liver was predicted from formation rate by recombinant CYP3A7, CYP3A7 expression level and the established ISEF value with average fold error of 1.25. Based on the intrinsic clearance of oxycodone measured in FLM, the fetal hepatic clearance (CL_h) at term was predicted to be 495 (range: 66.4–936) μL/min, a value that is >99% lower than the predicted adult liver CL_h. The predicted fetal hepatic extraction ratio was 0.0019 (range: 0.00003–0.0036). These results suggest that fetal liver metabolism does not quantitatively contribute to the total systemic clearance of oxycodone in pregnant women nor does it provide a barrier for limiting fetal exposure to oxycodone. Additionally, since CYP3A7 forms noroxycodone, an inactive metabolite, the metabolism in the fetal liver is unlikely to affect fetal opioid activity.

Keywords

Oxycodone; CYP3A7; Fetal hepatic clearance; intersystem extrapolation factor (ISEF); *in vitro*-to-*in vivo* extrapolation (IVIVE)

INTRODUCTION

The use of opioid analgesics close to term is associated with neonatal opioid withdrawal syndrome (NOWS) (1–3) and developmental delay in children exposed prenatally (4,5). In the United States, there has been a significant increase in the rate of opioid use during pregnancy (6), and the incidence of NOWS increased from 1.2 per 1000 births in year 2000 to 5.8 per 1000 births in 2012 (7). Yet, large scale epidemiological studies have not

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established a relationship between maternal opioid dose during pregnancy and associated fetal outcomes (8,9). While many reasons contribute to the lack of correlation, poor knowledge of the maternal-fetal disposition of opioid analgesics likely impacts the analysis. For example, it has been shown that buprenorphine interacts with P-glycoprotein (10), an efflux transporter that is expressed in the placenta potentially limiting fetal buprenorphine exposure. Similarly, fetal metabolism has been observed for morphine (11), codeine (12), and dextromethorphan (12,13), potentially decreasing fetal exposure to these drugs. Fetal metabolism can also result in formation of active metabolites. For example, in nonhuman primates morphine-6-glucuronide, an active metabolite of morphine, is formed in the fetus (14). The increased fetal exposure to an active metabolite formed locally in the fetus adds complexity to the maternal-fetal disposition and fetal pharmacological responses to opioids.

Oxycodone is an opioid commonly prescribed during pregnancy to treat moderate to severe pain (3,15). Oxycodone concentrations in umbilical cord are similar to those in maternal plasma following intravenous or subcutaneous doses of oxycodone given during early stages of labor (16,17), demonstrating that oxycodone crosses the placenta and distributes to the fetus. Despite the common usage of oxycodone during pregnancy, knowledge of the mechanisms of maternal-fetal disposition of oxycodone is limited, and whether fetal liver or the placenta limit exposure of the fetus to oxycodone or its metabolites is not known. In adults, oxycodone is extensively metabolized by CYP3A4 to form noroxycodone (fraction metabolized (f_m):0.4–0.5), an inactive metabolite, and by CYP2D6 to form oxymorphone (f_m:0.1–0.2), an active metabolite (18,19). Although CYP3A4 and CYP2D6 expression in fetal liver is very low (20,21), CYP3A7 is abundantly expressed in the fetal liver (20–22) and has been shown to metabolize many CYP3A4 substrates including midazolam, clarithromycin, carbamazepine, and alfentanil (23–25). In addition, the CYP3A4 mediated metabolic reactions of norcodeine formation from codeine (12) and 3-methoxymorphinan formation from dextromethorphan (12,13) in adult liver were also observed in FLM presumably mediated by CYP3A7. Based on these data of significant xenobiotic metabolic activities of CYP3A7 and FLM in metabolizing opioids that are CYP3A4 substrates, we hypothesized that CYP3A7 metabolizes oxycodone, and that the fetal liver contributes to the maternal-fetal disposition and metabolism of oxycodone. The goal of this study was to define the fetal liver metabolism of oxycodone and predict the quantitative importance of fetal liver metabolism of oxycodone.

MATERIALS AND METHODS

Fetal liver microsomes (FLM), adult human liver microsomes (HLM), and recombinant cytochrome P450 enzymes (CYPs)

Previously collected (26) banked fetal livers from 18 individuals (9 males, 7 females and 2 unidentified sex) were used. All tissues were from healthy pregnancies with no known maternal drug use and with previously published donor characteristics (26). Tissue collection was approved by the Institutional Review Board (IRB) of the University of Washington and was conducted according to the guidance of the Office of Human Research Protections (OHRP). The estimated gestational age at the time of fetal liver collection ranged from 85 to 137 days (26). To prepare FLM, approximately 100mg of fetal liver was

homogenized in 0.5mL 10mM phosphate buffer (pH 7.4) with 250mM sucrose and 0.2mM phenylmethylsulfonyl fluoride using an Omni Bead Ruptor 24 (Kennesaw, GA) at <4°C. The homogenates were centrifuged at 10,000 g for 20 min at 4°C and the supernatants were centrifuged at 100,000 g on Sorvall Discovery M150SE ultracentrifuge (Waltham, MA) for 1 hour at 4°C to collect microsomes. The microsomal pellets were reconstituted and stored at −80°C until experiments. Pooled FLM were prepared by pooling 14 individual FLMs. Human liver microsomes pooled from 50 donors were purchased from Sekisui Xenotech, LLC (Kansas City, KS). Membrane preparations of recombinant CYP2D6, CYP3A4, CYP3A5, and CYP3A7 coexpressed with P450 reductase and cytochrome b5 were purchased from Corning Life Sciences (Corning, NY). Recombinant, purified CYP3A7 expressed in E. Coli (27) and used as a calibration standard for CYP3A7 quantification in FLM was a gift from Emily E Scott, University of Michigan.

Quantification of CYP3A7 protein in FLM by HPLC-MS/MS

The CYP3A7 protein contents of the pooled FLM and 14 individual FLMs were quantified using a previously published HPLC-MS/MS method (28). Briefly, FLMs were diluted to 1mg/mL using 100mM ammonium bicarbonate buffer, 20μg of protein (20μL) were denatured and reduced with the addition of 4μL of 100mM dithiothreitol, 10μL of 2.6% w/v sodium deoxycholate and 10μL of 100mM ammonium bicarbonate buffer and the mixture was incubated at 95°C for 5min. The denatured protein was alkylated under yellow light using 4μL of 200mM iodoacetamide at room temperature for 20min. Thereafter, 0.4μg of trypsin (1:50) was added to digest microsomal protein at 37°C for 20h. The digestion time was adapted from (28) and confirmed to result in stable digestion from pooled FLM with no change in signal intensity between 20 and 24hrs in a digestion time-course from 4 to 24hrs. The digestion was stopped by the addition of 30μL 50% aqueous acetonitrile containing 0.1% formic acid and 15nM [$^{13}C_6{}^{15}N_2$]-lysine labelled peptide as internal standard. The samples were centrifuged at 20,000g for 45min. Calibration standards were prepared by spiking known amount of purified soluble CYP3A7 protein (expressed in *E. coli*) to pooled HLM (confirmed to lack detectable CYP3A7 expression). Seven calibration concentrations at 40–800pmol/mg protein and three quality control samples along with blank controls were included in each assay. The experiment was performed in singlet on three different days and the protein concentration of each FLM is reported as the arithmetic mean and the standard deviation of the data from three different days.

Target peptide (FNPLDPFVLSIK) concentrations were measured using an AB Sciex Triple Quad™ 5500+ QTRAP mass spectrometer (Foster City, CA) equipped with an Agilent 1290 UHPLC (Santa Clara, CA) and a Phenomenex Aeris™ PEPTIDE XB-C18 LC column (1.7μm, 50×2.1mm) with a Phenomenex SecurityGuard™ C18-Peptide cartridge (sub-2μm, 2.1mm) (Torrance, CA). A 16-min gradient was employed using (A) water containing 0.1% formic acid and (B) acetonitrile containing 0.1% formic acid at a flow rate of 0.4mL/min. The target peptide and its labelled internal standard were detected by electrospray ionization operated in positive ion mode and two product ions were monitored (m/z 695>918, 695>565 and m/z 699>927, 699>569 for target and heavy labelled peptide, respectively) to confirm the analytes and that the most sensitive product ion was used for quantification (m/z

695>565 and m/z 699>569 for target and heavy labelled peptide, respectively). The lower limit of quantification (LLOQ) for CYP3A7 was 20pmol/mg protein.

In vitro incubations

All incubation experiments were done in potassium phosphate buffer (pH 7.4) at 37°C, were initiated by addition of NADPH and included no NADPH negative controls. Incubations were conducted in duplicates on three different days except for four individual FLMs for which the oxycodone incubations were done in duplicates on two different days due to limited availability of the fetal liver. Incubations were quenched by addition of two parts of ice-cold methanol containing noroxycodone-d_3 and oxymorphone-d_3 as internal standards, vortexed and centrifuged at 16,100g, and supernatants were stored at −20°C until HPLC-MS/MS analysis. Oxycodone, noroxycodone, oxymorphone, noroxycodone-d_3 and oxymorphone-d_3 were purchased from Millipore Sigma (St Louis, MO).

Noroxycodone and oxymorphone formation kinetics was characterized in recombinant CYP microsomes, pooled HLM, and pooled FLM. Under conditions of protein and time linearity, oxycodone (1, 2, 5, 15, 30μM) was incubated with recombinant CYP2D6 (5pmol/mL), CYP3A4 (1pmol/mL), CYP3A5 (5pmol/mL), CYP3A7 (5pmol/mL), pooled HLM (0.05mg/mL), or pooled FLM (0.1mg/mL) for 15min. Additionally, 6β-OH-testosterone formation kinetics was characterized in recombinant CYP3A7 and pooled FLM. Testosterone (5, 10, 15μM) was incubated with recombinant CYP3A7 (10pmol/mL) and pooled FLM (0.2mg/mL) for 15min. To further evaluate the intrinsic capacity and interindividual variability of fetal liver metabolism of oxycodone, noroxycodone and oxymorphone formation was measured in FLM from 18 individual fetal livers. Oxycodone (5μM) was incubated in FLM (0.2mg/mL) for 30min. The percent contribution of CYP3A to metabolite formation in FLMs and HLM was determined in incubations with 1μM of ketoconazole to inhibit CYP3A activity. To measure CYP3A activity in each FLMs (n=18), 6β-OH-testosterone formation from 35μM testosterone was measured.

HPLC-MS/MS Analysis

Metabolite concentrations were measured using an AB Sciex 6500 qTrap Q-LIT mass spectrometer (Foster City, CA) equipped with a Shimadzu UPLC XR DGU-20A5 (Columbia, MD) and a Kinetex® EVO C18 LC column (2.6μm, 100×2.1mm) with a Phenomenex SecurityGuard™ EVO-C18 cartridge (sub-2μm, 2.1mm) (Torrance, CA). For noroxycodone and oxymorphone analysis, gradient elution (0.35 mL/min) with (A) 20mM ammonium formate and (B) 50:50 v/v acetonitrile and methanol starting at 0% (B) for 1min, increased to 25% (B) over 2min, kept at 25% (B) for 1.5min, increased to 100% (B) over 0.5min and kept at 100% (B) for 0.5min before returning to initial conditions over 0.5min was used. An 8-min gradient using the same mobile phases (flow 0.3mL/min) was used for 6β-OH-testosterone analysis. The gradient was from 5% (B) for 1min, increased to 100% (B) over 3min and kept at 100% (B) for 0.5min, returning to initial conditions over 0.1min. The analytes were detected by positive ion electrospray ionization. Three product ions were monitored to confirm the identity of analytes and the most sensitive product ion was used to quantify each analyte (noroxycodone m/z 302>187, oxymorphone m/z 302>227, 6β-OH-testosterone m/z 305>269). The LLOQ of noroxycodone and oxymorphone were 1nM. For

noroxycodone and oxymorphone, calibration standards (1–300nM) were prepared in phosphate buffer. Quality controls (QCs) (3, 30, 150 and 240nM) were included in every run and the intraday and interday precision and accuracy were within 10% for both analytes throughout. For 6β-OH-testosterone (calibration range 1–300nM), LLOQ was 10nM, intraday and interday precision were within 15% and accuracy within 10% for quality control (QC) concentrations 16, 130 and 230nM included in every LC-MS/MS run.

Data analysis

The metabolite formation rates were normalized to either pmol CYP or mg microsomal protein. Straight lines were fitted to the noroxycodone formation rate over oxycodone concentration to estimate the intrinsic clearances (CL_{int}) (29) of noroxycodone formation by CYP3A4, CYP3A7, pooled HLM, and pooled FLM. Similarly, straight lines were fitted to the 6β-OH-testosterone formation rate over testosterone concentration to estimate the CL_{int} of 6β-OH-testosterone formation by CYP3A7 and pooled FLM ($CL_{int,FLM}$). Michaelis-Menten equation was fitted to the oxymorphone formation rate over oxycodone concentration data to estimate the k_{cat}, K_m, and CL_{int} of oxymorphone formation by CYP2D6. All results are reported as the arithmetic means and standard deviations of data from three different days. The intersystem extrapolation factor (ISEF) for CYP3A7 for 6β-OH-testosterone formation was calculated using equation 1:

$$ISEF = \frac{CL_{int,\,FLM}}{CL_{int,\,CYP3A7} \times CYP3A7\,abundance} \quad (1)$$

where CYP3A7 abundance is the measured CYP3A7 expression in the pooled FLM. The calculated ISEF was applied to predict the $CL_{int,FLM}$ of noroxycodone formation in pooled FLM from the CL_{int} of noroxycodone formation in recombinant CYP3A7 by multiplying the CL_{int} of CYP3A7 with the CYP3A7 expression (pmol CYP/mg protein) and the calculated ISEF. The $CL_{int,FLM}$ of noroxycodone formation were predicted similarly for each individual FLM using the ISEF value calculated from the 6β-OH-testosterone formation in each respective FLM.

The $CL_{int,FLM}$ in 16 livers (two FLMs showed no noroxycodone formation) was calculated as the noroxycodone formation rate divided by the oxycodone concentration. The $CL_{int,FLM}$ of 6β-OH-testosterone formation was calculated as the formation rate divided by the testosterone concentration. The formation rate, intrinsic clearance, and ketoconazole % inhibition were reported as the arithmetic means of data from three different days or as the average of two days when limited data was available. The difference between the mean noroxycodone formation rate in the presence and absence of ketoconazole for individual FLMs were tested using the paired t-test. Correlation between the mean CL_{int} of noroxycodone formation and the mean CL_{int} of 6β-OH-testosterone formation or the mean CYP3A7 expression in each FLM were tested by linear regression.

All calculations were performed using Microsoft Excel for Microsoft 365 (Redmond, WA). Graphical presentation of results and statistical analysis were done using GraphPad Prism version 8 (San Diego, CA).

In vitro-to-*in vivo* extrapolation (IVIVE)

Fetal hepatic clearance (CL_h) and extraction ratio (ER) of oxycodone were first predicted for each of the 16 donor livers that showed noroxycodone formation. To do this, the unbound intrinsic clearance for the fetal liver ($CL_{int,u}$) was calculated from $CL_{int,FLM,}$ the microsomal protein per gram of liver (MPPGL) yield, and the estimated fetal liver weight using equation 2:

$$CL_{int,u} = CL_{int,FLM} \times MPPGL \times liver\,weight \quad (2)$$

The measured MPPGL was 8–33mg/g. The liver weight was calculated using the reported (30) equation 16.6–2.9*GA+0.143GA^2 for fetal liver volume (mL) assuming fetal liver density of 1g/mL and based on the reported (26) gestational age in weeks (GA) in each donor resulting in liver weight 2.2–14.2g. The fetal liver blood flow (Q_h) was calculated as $Q_{Umbilical\_Vein}$+$Q_{fetal\_portal\_vein}$ -$Q_{ductus\_venosus}$ (resulting range 1.8–4.4L/h) using the published (30) equations for $Q_{Umbilical\_Vein}$=0.647–0.227*GA+0.0179*GA^2 (resulting range 0.5–3.1L/h). $Q_{ductus\_venosus}$=2.05–0.297*GA + 0.0116*GA^2 (resulting range 0.2–0.7L/h), and $Q_{fetal\_portal\_vein}$=0.714+0.0489*GA+0.0008*GA^2 (resulting range 1.4–2.0L/h).

Fetal CL_h was then calculated using equation 3 (the well-stirred model (31)):

$$CL_h = \frac{\frac{f_u}{BP\,ratio} \times CL_{int,u} \times Q_h}{\frac{f_u}{BP\,ratio} \times CL_{int,u} + Q_h} \quad (3)$$

where f_u is the unbound fraction in fetal plasma (same as adult, f_u=0.55) and BP ratio is the blood to plasma concentration ratio (same as adult, BP ratio=1.3, (32)). The extraction ratio (ER) by the fetal liver was calculated as CL_h/Q_h.

To extrapolate the findings from early gestation to later gestational ages, fetal CL_h and ER from gestational week 12 to 40 were predicted using equations 2 and 3 with the average $CL_{int,FLM}$*MPPGL measured in the 16 individual fetal livers used in equation 2 together with the calculated fetal liver weight and blood flows for each GA incorporated into equations 2 and 3. The CYP3A7 expression and hence $CL_{int,FLM}$ was assumed to be independent of gestational age as CYP3A7 expression has been shown to be relatively constant from early to late gestation based on mRNA expression, protein expression, and enzymatic activity data (20,22). The upper and lower limits of the CL_h and ER from gestational week 12 to 40 were predicted using the maximum and minimum values obtained for $CL_{int,FLM}$*MPPGL of the 16 fetal livers.

Adult hepatic clearance (CL_h) and extraction ratio (ER) of oxycodone were also predicted using the well stirred model with Q=1.5L/min and incorporating the sum of the pooled HLM measured noroyxoconde and oxymorphone CL_{int}'s multiplied by MPPGL of 39.8mg/g and liver weight of 1,648g.

RESULTS

Recombinant CYP3A7 metabolized oxycodone to noroxycodone similar to recombinant CYP3A4, while no quantifiable noroxycodone formation was observed by CYP3A5 (Fig 1a). With CYP3A4 and CYP3A7, noroxycodone formation was linear from 1 to 30μM oxycodone suggesting that the K_m's of oxycodone with CYP3A4 and CYP3A7 are much greater than 30μM (Fig 1b,c). The intrinsic clearance (CL_{int}) of noroxycodone formation by CYP3A7 (0.042±0.007μL/min/pmol CYP) was 89% lower than that by CYP3A4 (0.38±0.07μL/min/pmol CYP). No oxymorphone formation was observed with any of the recombinant CYP3A enzymes, but recombinant CYP2D6 formed oxymorphone and not noroxycodone (Fig 1a). The K_m of oxycodone with CYP2D6 was estimated to be 14±1.8 μM. The CL_{int} of oxymorphone formation by recombinant CYP2D6 (0.27±0.05μL/min/pmol CYP) was 29% lower than that of noroxycodone formation by recombinant CYP3A4 (Fig 1d). Based on the recombinant enzyme kinetics, a reported ISEF value for CYP3A4 (ISEF: 0.15) (33), and reported CYP3A4 expression in HLM (78pmol/mg protein) (34), noroxycodone formation CL_{int} in HLM ($CL_{int,HLM}$) was predicted to be 4.4μL/min/mg protein. Since no published ISEF value has been reported for CYP3A7, the ISEF value for CYP3A7 was estimated in this study. To determine the CYP3A7 ISEF value for FLMs, CYP3A7 protein expression was measured in pooled FLM and in 14 individual FLMs. The measured CYP3A7 expression was 359±41pmol/mg protein in pooled FLM (Fig 2) and ranged from 191 to 409pmol/mg protein in the individual FLMs (mean 326±74). The 6β-OH-testosterone formation CL_{int} was used for ISEF value estimation and measured in recombinant CYP3A7 to be 0.072±0.002μL/min/pmol CYP, in pooled FLM to be 1.13±0.21μL/min/mg protein, and in 14 individual FLMs to be between 0.035 and 1.95 μL/min/mg protein (mean 0.77±0.51). The ISEF value of CYP3A7 was calculated to be 0.044 based on the pooled FLMs and to range from 0.016 to 0.066 (mean 0.036±0.016) in the individual FLMs using single substrate 6β-OH-testosterone as a probe. The calculated ISEF of CYP3A7 was then applied to the mean noroxycodone formation CL_{int} by recombinant CYP3A7 multiplied by the CYP3A7 expression in pooled FLM and the $CL_{int,FLM}$ of noroxycodone formation in pooled FLM was predicted to be 0.66μL/min/mg protein, 85% lower than the predicted $CL_{int,HLM}$. The corresponding individual FLM ISEF and CYP3A7 expression values were used to predict the $CL_{int,FLM}$ of each individual FLM and $CL_{int,FLM}$ were predicted to range from 0.15 to 1.13μL/min/mg protein (mean 0.50±0.27). Similarly, the oxymorphone formation $CL_{int,HLM}$ was predicted to be 0.44μL/min/mg protein using the reported ISEF of 0.18 for CYP2D6 (33) and the reported CYP2D6 expression data in HLM (9.1pmol/mg protein) (34). No oxymorphone was predicted to form in FLM based on the minimal CYP2D6 expression reported in FLM (13,28,35).

Both noroxycodone and oxymorphone formation were observed in pooled HLM (Fig 1e). The noroxycodone formation was linear from 1 to 30μM oxycodone while the oxymorphone formation was saturated at high oxycodone concentrations consistent with observations with recombinant CYP3A4 and CYP2D6 (Fig 1f). The $CL_{int,HLM}$ for noroxycodone formation in pooled HLM was 5.8±0.4μL/min/mg protein and the $CL_{int,HLM}$ for oxymorphone formation in pooled HLM was 0.47±0.06μL/min/mg protein (Fig 1h) which were 1.32-fold and 1.07-

fold of the respective $CL_{int,HLM}$ values predicted from recombinant CYP3A4 and CYP2D6. In contrast, only noroxycodone and no oxymorphone formation was observed in pooled FLM (Fig 1g). The $CL_{int,FLM}$ for noroxycodone formation in pooled FLM was 0.60±0.07μL/min/mg protein which was only 9% lower than that predicted from recombinant CYP3A7.

Noroxycodone formation was characterized in 16 individual FLMs. The mean noroxycodone formation $CL_{int,FLM}$ in the individual FLMs was 0.53±0.30 (range: 0.064–1.0)μL/min/mg protein (Fig 3a). When FLMs were incubated with ketoconazole, a CYP3A inhibitor, noroxycodone formation was decreased by 87±9%, supporting the notion that noroxycodone formation in FLMs is primarily, and nearly entirely, mediated by CYP3A. The noroxycodone formation $CL_{int,FLM}$ correlated significantly with CYP3A7 expression ($p=0.0078$) and 6β-OH-testosterone formation $CL_{int,FLM}$ ($p<0.0001$), a CYP3A probe reaction, (36) in the individual FLMs (Fig 3b,c), demonstrating that oxycodone metabolism in fetal liver is predominantly mediated by CYP3A7. The observed $CL_{int,FLM}$ for noroxycodone formation correlated significantly with that predicted from recombinant CYP3A7 in the individual FLMs ($p<0.0001$) (Fig 3d) and the observed $CL_{int,FLM}$ were within 83–177% (mean 127±24%) of the predicted $CL_{int.FLM}$.

The fetal hepatic clearance (CL_h) and extraction ratio (ER) for oxycodone were predicted based on the noroxycodone formation data obtained for each fetal liver tested and the known fetal liver size and hepatic blood flow at different gestational stages. The predicted values for fetal CL_h and ER were 0.020±0.012 (range: 0.002–0.044)mL/min and 0.00042±0.00021 (range: 0.00005–0.00081), respectively for the fetal livers from early gestation (85–137 days) (Fig 4). The predicted fetal liver CL_h and ER at term (GA 40weeks) were 0.496mL/min (range: 0.066 – 0.936) and 0.0019 (range: 0.00003–0.0036), respectively (Fig 4). In comparison, the predicted adult CL_h was 154mL/min, more than 300-fold greater than that of the fetus at term, reflecting the substantial difference in the size of adult and fetal liver and the higher catalytic activity of CYP3A4 in comparison to CYP3A7. The predicted adult hepatic ER was 0.10, more than 50-fold greater than that of the fetal liver.

DISCUSSION

Fetal metabolism of drugs has been extensively studied for more than 60 years (37,38), and the anatomical and biochemical functions of the fetal liver have been shown to be different from adult liver (37,39). For example, the fetal liver receives a significant fraction of its blood supply from the placenta in addition to the portal vein and hepatic artery (40), the morphology of the endoplasmic reticulum changes over gestation (37), and the zonation of drug metabolizing enzyme expression along the acinus is less apparent than in adult liver (39). Drug metabolizing enzyme expression in the fetal liver is also substantially different from adult liver. It has been shown through gene expression analysis (13,20,35), protein quantification (21,28), and measurements of enzyme activities (12,13,21) that CYP3A7 is the most abundant CYP expressed in the fetal liver while the expression of other CYPs including CYP3A4 and CYP2D6 in fetal liver is minimal (20–22,28,35). Additionally, FMO1 and GSTP proteins have been shown to be abundantly expressed in fetal liver at levels comparable to FMO3 and GSTM in adult liver (41). Of the conjugation enzymes,

SULTs and UGTs have been detected in the fetal liver (41). For example, UGT2B7, UGT2B15, and UGT2B17 are expressed in the fetal liver but at levels of about 10% of the adult liver (41,42). Taken together these findings show that the fetal liver possesses its own unique complement of drug metabolizing enzymes and may have different metabolic capacity and specificity than the adult liver.

This study clearly shows that CYP3A7, the most abundantly expressed CYP enzyme in the fetal liver (20,21), metabolizes oxycodone. Oxycodone is N-demethylated by CYP3A4 to noroxycodone, a major metabolite, and O-demethylated by CYP2D6 to oxymorphone, an active metabolite (18). These metabolic pathways of oxycodone by CYP3A4 and CYP2D6 were confirmed in this study and CYP3A7 was found to N-demethylate oxycodone similar to CYP3A4. This finding is in agreement with the shared substrate specificity between CYP3A4 and CYP3A7 (23). However, in contrast to a previous report (18), no noroxycodone formation by CYP3A5 was observed in this study despite the fact that a positive control (testosterone) was metabolized by the recombinant CYP3A5 at a rate similar to what has been previously observed (36) (data not shown). The difference between the studies could be due to different expression systems used (i.e. baculovirus-infected insect cells in this study compared to human lymphoblastoid cells in the previous study) or the coexpression of reductase with the CYPs used in this study. The observation that oxycodone is metabolized by both CYP3A4 and CYP3A7 but not by CYP3A5 is somewhat surprising, since CYP3A5 has overlapping substrate specificity with CYP3A4 and CYP3A7 (23,43), and the CL_{int} of CYP3A5 is often higher than CYP3A7 (23).

The CYP3A7 protein expression in FLM quantified in this study (191–409pmol/mg protein) is consistent with that observed before based on CYP3A7 activity towards metabolizing DHEA (201–311pmol/mg protein) (21) while higher than that quantified by LC-MS/MS (10–160pmol/mg protein) (28). This discrepancy is likely due to the difference in calibration standards. In this study purified CYP3A7 protein spiked into HLMs was used as a calibration standard while surrogate peptide was used in the previous study. To our knowledge, this is the first study to use purified CYP3A7 protein to quantify CYP3A7 expression in microsomes. The use of whole protein as a standard digested in the HLM matrix will correct for digestion efficacy and variability and is less likely to underpredict true protein expression as shown (44). Although CYP2D6 expression was not measured in this study, multiple studies have reported that CYP2D6 is minimally expressed in fetal liver (13,28,35). As expected from the CYP3A7 and CYP2D6 expression patterns, this study showed that noroxycodone was formed in FLM, but oxymorphone was not. Similarly, CYP3A7 mediated metabolism has been observed for other opioids in FLM. For example, norcodeine formation from codeine, 3-methxymorphinan formation from dextromethorphan, and norethylmorphine formation from ethylmorphine have been observed in FLM (13,45). On the other hand, CYP2D6 mediated metabolism, such as morphine formation from codeine or dextrorphan formation from dextromethorphan has not been observed in FLM when tested (12). With the finding of lack of oxymorphone formation by fetal liver in this study it may be concluded that fetal liver metabolism does not increase the risk of fetal exposure to active opioid metabolites formed by CYP2D6.

All of the data collected in this study support the conclusion that CYP3A7 is the main enzyme catalyzing noroxycodone formation from oxycodone in FLMs. This conclusion is supported by the nearly complete inhibition of noroxycodone formation in the FLM by ketoconazole, a CYP3A7 inhibitor (27), and tight correlations of noroxycodone formation clearance with 6β-OH-testosterone formation clearance and CYP3A7 expression in each FLM (36). Although the role of CYP3A4 and CYP3A5 cannot be ruled out in the above experiments, the combination of the observations in this study that show minimal noroxycodone formation activity by CYP3A5 and previous studies that show that expression of CYP3A7 in fetal liver is more than 100-fold greater than CYP3A4 (20) suggest that CYP3A7 is the main enzyme responsible for noroxycodone formation in FLM.

It has been shown for several CYPs that the activities measured in recombinant CYPs cannot be directly scaled to the adult or fetal liver without the consideration of the ISEF or a relative activity factor (RAF) (33,34). This study is the first to establish an ISEF value to scale CYP3A7 activity to FLM enabling predictions of FLM metabolism of variety of drugs based on recombinant enzyme data. However, ISEF values can vary between substrates and they are sensitive to the lot of enzyme, cytochrome b5 and reductase expression and expression system (34) and as such may vary between sources or even with gestational age if reductase or b5 expression is gestational age dependent. It should be noted that the fetal liver CL_h for GA 20–40weeks shown here is based on extrapolation and assuming minimal changes in intrinsic activity of CYP3A7 or changes in f_u or BP ratio with gestational age. The fact that these parameters may change with gestational age yields some uncertainty to the CL_h and ER values predicted beyond 20 weeks. The ISEF value established here based on 6β-OH-testosterone formation was applied to predicting noroxycodone formation $CL_{int,FLM}$ at early gestation fetal liver from activity measured with recombinant CYP3A7. The observed noroxycodone formation $CL_{int,FLM}$ were 83–177% of those predicted suggesting that the CYP3A7 expression is a major contributor to the observed variability in noroxycodone formation CL_{int} in FLM. Notably, there was a 4-fold range of ISEF values (0.016–0.066) calculated for the individual FLMs indicating intrinsic factors such as membrane environment and reductase or b5 expression in the individual livers also contribute to the interindividual variability independent of CYP3A7 expression. Without an ISEF (i.e. ISEF=1), the scaled noroxycodone formation CL_{int} in FLM overpredicts the observed $CL_{int,FLM}$ of noroxycodone 25-fold. There are examples of drugs commonly used during pregnancy that are metabolized by CYP3A7, including midazolam, clarithromycin, alfentanil, glyburide and carbamazepine (20,23–25,28). The catalytic activity of CYP3A7 to metabolize these drugs has been shown to be up to 26% of that of CYP3A4. The establishment and further refinement of an ISEF value for CYP3A7 using several substrates together with the robust measurement of CYP3A7 protein expression in FLMs, provides a valuable tool to quantitatively predict fetal liver metabolism for these drugs and other CYP3A substrates administered to pregnant women without the need to acquire fetal liver tissue.

The quantitative *in vitro*-to-*in vivo* extrapolation (IVIVE) of fetal liver clearance of oxycodone suggests that the fetal liver plays a minor role in the maternal-fetal disposition of oxycodone. An obvious factor for the insignificant contribution by the fetal liver is that the fetal liver weight is less than 10% of that of the adult liver (31), and hence, the fetal liver

CL_h is predicted to be small when compared to the adult CL_h as demonstrated previously for retinoic acid (26). Another factor is the low intrinsic capacity of the fetal liver to metabolize oxycodone. The interindividual variability observed in the CL_{int} of noroxycodone formation in individual FLM was considerable (16-fold), consistent with the observed variability in 6β-OH-testosterone formation and CL_{int} of retinoic acid metabolism reported previously (26). However, this interindividual variability has a minor impact on the systemic clearance of oxycodone in pregnant women, as shown by the predicted ranges of fetal CL_h in comparison to the adult CL_h. Since the fetal liver is acting as a first-pass organ for the fetus that half of the blood coming from the placenta passes through the fetal liver before entering the fetal circulation, the fetal hepatic ER can be used to predict how well the fetal liver protects the fetus from exposure to xenobiotics. The extremely low predicted fetal hepatic ER of oxycodone suggest that the fetal liver does not act as a barrier to protect the fetus from exposure to oxycodone that passes through the placenta. Similar to the predicted low fetal hepatic ER for oxycodone, the fetal hepatic ERs were predicted to be <0.05 for other drugs with available FLM metabolism information including codeine (12), dextromethorphan (13), and glyburide (28). These predictions further support the results of this study that the fetal liver does not act as a barrier to protect the fetus from exposure to xenobiotics. Based on the data obtained in this study a xenobiotic with no protein binding and BP ratio=1 would need to have an $CL_{int,FLM}$ of at least 45μL/min/mg protein to have an ER>0.3 by fetal liver that could provide some protection to the fetus from maternal exposures.

CONCLUSION

This study demonstrates that CYP3A7 in the fetal liver metabolizes oxycodone but the role of the fetal liver is minimal in the maternal-fetal disposition of oxycodone. These results complement the clinical and preclinical studies performed to study the maternal-fetal disposition of opioids during pregnancy (16,17,46–50). The findings of this study are in agreement with previous work showing that the quantitative contribution of fetal hepatic clearance to the maternal-fetal disposition of CYP3A7 substrates is predicted to be minimal and highlights the need to quantitatively scale CYP3A7 activity to fetal liver to assess significance of fetal liver metabolism. An ISEF value for CYP3A7 was established together with CYP3A7 expression levels in FLMs to provide tools to predict fetal liver metabolism from recombinant enzyme data. Altogether, this study suggests that fetal liver is unlikely to generally contribute to the maternal-fetal disposition xenobiotics unless the CYP3A7 catalytic activity is significantly greater than that of CYP3A4.

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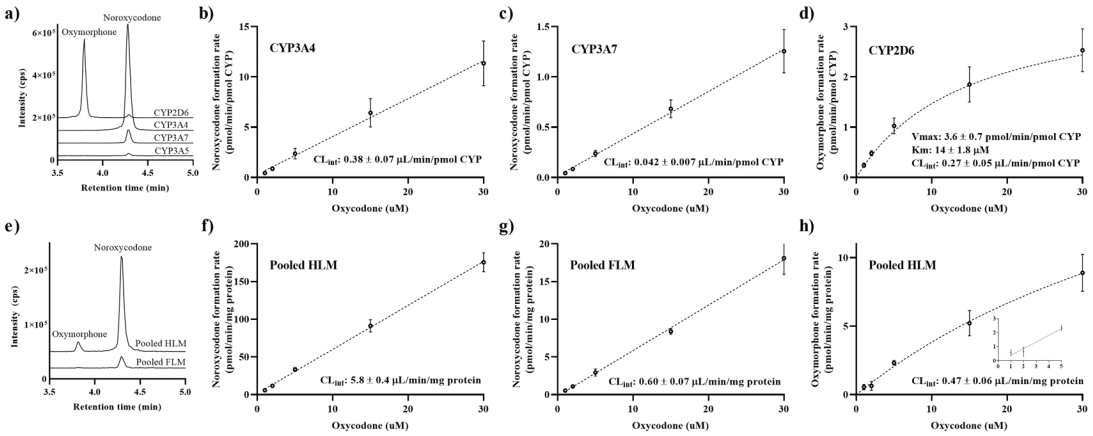

Figure 1. Noroxycodone and oxymorphone formation kinetics in recombinant CYPs, pooled HLM (50 donors), and pooled FLM (14 donors).

HPLC-MS/MS chromatograms showing noroxycodone and oxymorphone formation from 5 µM oxycodone after 15 min incubation with 5 pmol/mL CYP2D6, CYP3A4, CYP3A5, and CYP3A7 are shown in (a). Metabolite formation rate as a function of oxycodone concentration for noroxycodone formation by recombinant CYP3A4 (b) and CYP3A7 (c), and for oxymorphone formation by recombinant CYP2D6 (d) are shown with CL_{int} or Michaelis Menten parameters determined for the metabolite formation listed in inset. HPLC-MS/MS chromatograms noroxycodone and oxymorphone formation from 5 µM oxycodone after 15 min incubation with 0.1 mg/mL pooled HLM and 0.1 mg/mL pooled FLM are shown in (e). Metabolite formation rate as a function of oxycodone concentration for noroxycodone formation with pooled HLM (f) and pooled FLM (g), and for oxymorphone formation pooled HLM (h) are shown with the estimated CL_{int} values for the metabolite formation listed in inset. Oxymorphone formation in pooled HLM was not linear but V_{max} and K_m could not be reliably estimated from 1 to 30 µM oxycodone and CL_{int} of oxymorphone formation was estimated using the initial concentration from 1 to 5 µM oxycodone (linear regression shown in inset). Mean and standard deviation of intrinsic clearance (CL_{int}), Vmax, and K_m reported were calculated from three different experiments conducted on separate days.

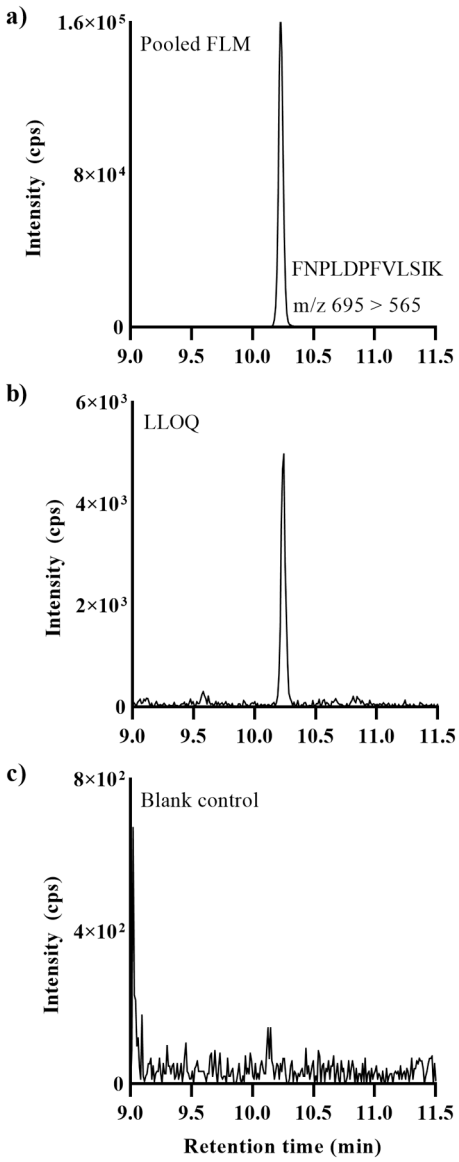

Figure 2. HPLC-MS/MS chromatograms of CYP3A7

in a) pooled FLM, b) HLM spiked with 20 pmol CYP3A7 per mg microsomal protein (LLOQ), and c) blank HLM matrix control. The targeted peptide sequence and the MRM transitions are listed as insets in panel a.

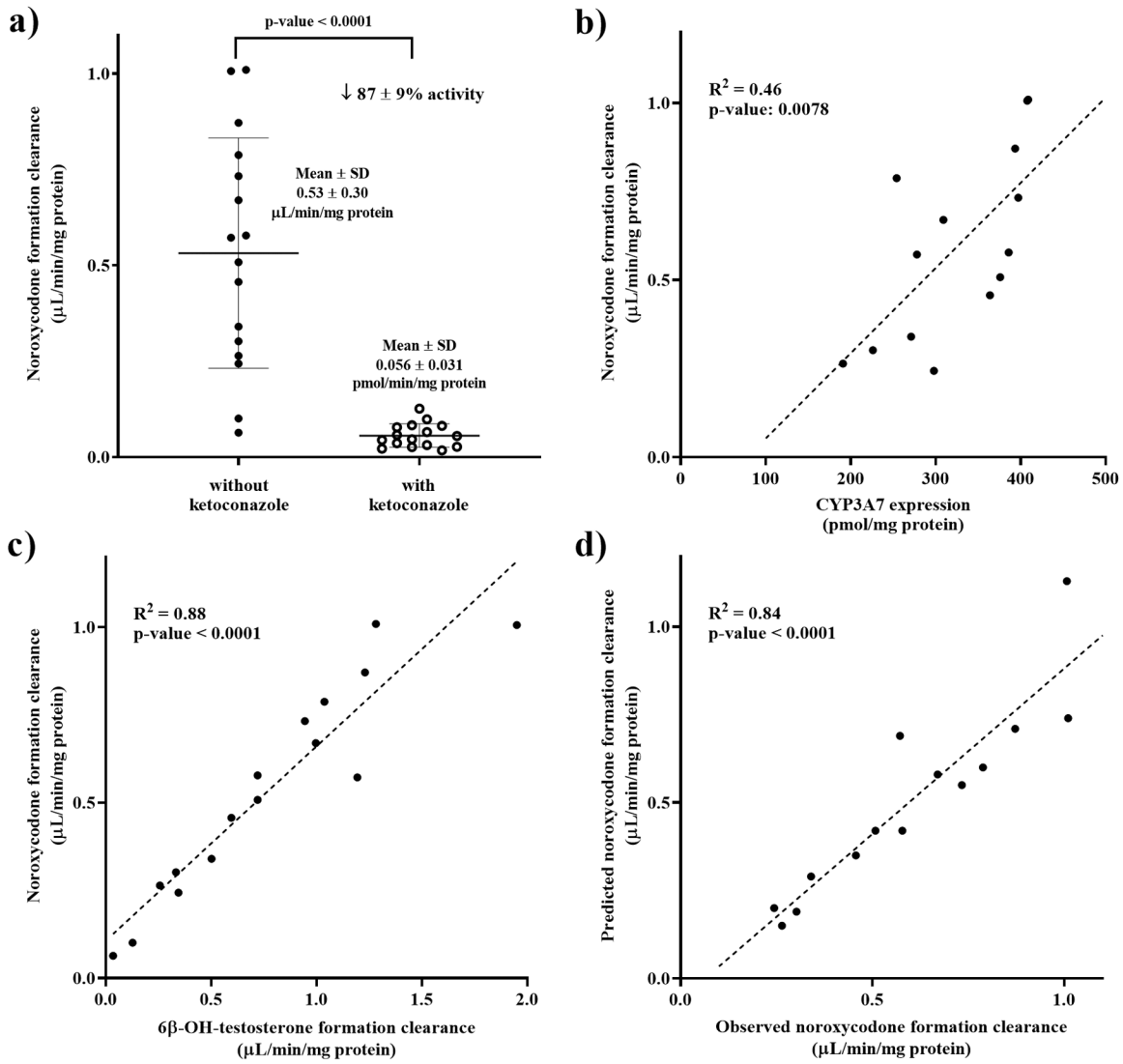

Figure 3. Characterization of noroxycodone formation from oxycodone in FLMs.

a) Noroxycodone formation rate in the absence (filled circles) and presence (open circles) of 1 µM of ketoconazole in the individual FLMs (n=16). b) Correlation between noroxycodone formation clearance with CYP3A7 protein expression in the individual FLMs (R^2: 0.46) with a slope significantly different from zero (p-value: 0.0078) (n=14). The mean CYP3A7 expression in the FLMs was 326 ± 74 pmol/mg protein. c) Correlation between noroxycodone and 6β-OH-testosterone formation clearances in the individual FLMs. The mean 6β-OH-testosterone $CL_{int, FLM}$ was 0.68 ± 0.54 µL/min/mg protein and the 6β-OH-testosterone $CL_{int, FLM}$ correlated with the noroxycodone $CL_{int, FLM}$ (R^2: 0.88) with a slope significantly different from zero (p-value <0.0001) (n=16). d) Correlation between the predicted noroxycodone formation clearance using ISEFs calculated for individual FLMs and the measured CYP3A7 expression in individual microsomes with the observed noroxycodone formation clearance in the individual FLMs (R^2: 0.84) with a slope significantly different from zero (p-value <0.0001) (n=14).

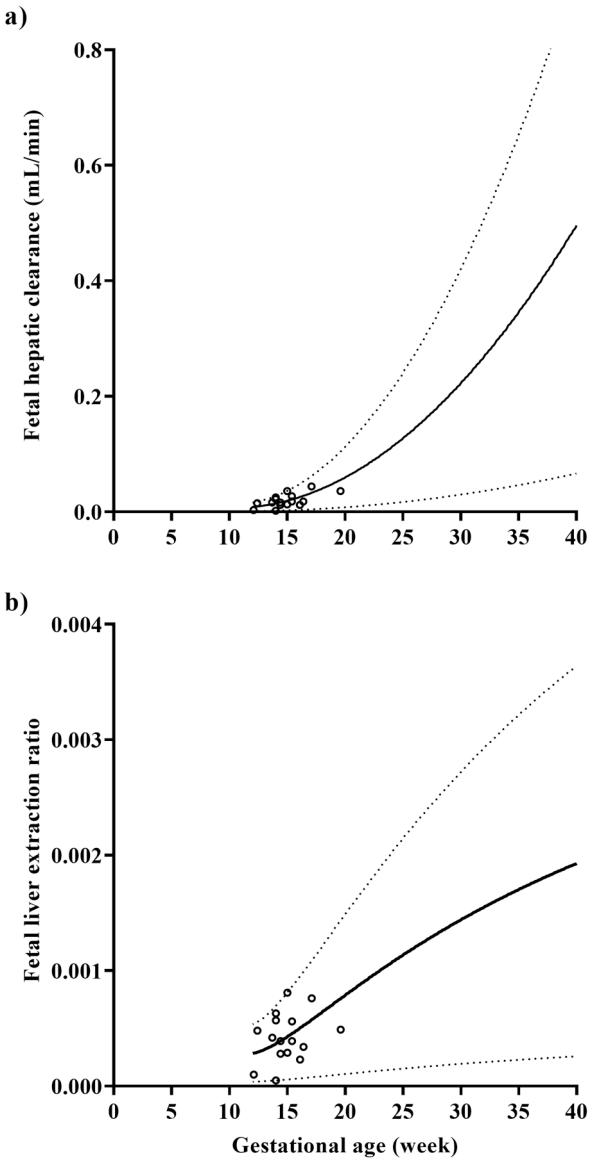

Figure 4. Predicted a) fetal hepatic clearance (CL_h) and b) fetal liver extraction ratio (ER) from gestational week 12 to gestational week 40.

The open circles represent the predicted fetal CL_h and fetal liver ER for each individual donor liver. The solid lines represent the CL_h and ER extrapolated using the mean estimated fetal liver intrinsic clearance (CL_{int}), the dotted lines represent CL_h and ER extrapolated using the range of the estimated CL_{int}, in the fetal livers tested.